

# Wheat germ agglutinin but not concanavalin A modulates protein kinase C-mediated phosphorylation of red cell skeletal proteins

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Human red blood cells contain protein kinase C (PKC) which acts exclusively on the membrane skeletal proteins band 4.1, band 4.9 and adducin. PKC activity can be stimulated by the addition of the phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate to intact cells. Phosphorylation of band 4.1 by PKC in vitro results in a dramatic reduction in band 4.1 binding to spectrin and actin, as well as to the cytoplasmic domain of band 3. Here we show that the lectin wheat germ agglutinin (WGA), which binds to the extracellular domain of glycophorin results in the inhibition of PKC catalyzed phosphorylation of band 4.1, band 4.9 and likely adducin as well. The lectin concanavalin A, which binds to band 3 was without effect. Our results suggest that the binding of WGA to glycophorin results in a major rearrangement of the membrane skeletal network which correlates with reduced phosphorylation of membrane skeletal proteins by PKC.

Protein kinase C; Phosphorylation; Erythrocyte; Band 4.1

## 1. INTRODUCTION

The membrane skeleton of the red blood cell is a dense self-assembled network consisting principally of the proteins spectrin, band 4.1, actin and band 4.9 [1,2] and is attached to the red cell membrane via several types of associations [2–6]. Recent evidence suggests that many of these associations may be regulated by changes in the state of phosphorylation of skeletal proteins [7–9]. Considerable evidence suggests that the associations of membrane skeletal proteins with themselves and with integral membrane components combined with the properties of the lipid bilayer are responsible for the characteristic shape and mechanical properties of the red cell [10].

The linkage of the membrane skeleton to the transmembrane proteins band 3 and glycophorin [3,4] suggest the possibility that agents which affect the extracellular domains of these proteins may perturb or alter the membrane skeleton. Such transmembrane signalling has in fact been documented [11,12]. More recently, we have shown that proteolysis of band 3 or neuraminidase treatment of glycophorin in intact cells

inhibits PKC-mediated phosphorylation of membrane skeletal proteins [13]. These and other results [14] suggest that extracellular signals may be transmitted via glycophorin or band 3 to the cytoplasmic surface of the membrane, resulting in major rearrangements of the entire membrane skeletal network. Correlations between changes in membrane skeletal organization and protein kinase activity are of great interest because protein phosphorylation likely plays a key role in regulating protein associations in the membrane skeleton. To explore the relationship between extracellular signals and protein kinase activity further, we have investigated the effects of lectins on PKC-dependent phosphorylation of red cell skeletal proteins. We used WGA, which binds specifically to glycophorin [15], and Con A, which binds to band 3 [16], as our test ligands.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Human blood was obtained from the blood bank of St. Elizabeth's Hospital of Boston less than two weeks after drawing. TPA, WGA, Con A, DFP, PMSF and adenosine were obtained from Sigma. [ $\gamma$ - $^{32}$ P]ATP,  $^{32}$ PO<sub>4</sub> and  $^{125}$ I-labeled Bolton Hunter reagent were obtained from New England Nuclear. All other chemicals were of reagent grade.

### 2.2. Phosphorylation in whole cells

Intact red cells were metabolically labeled for 4 h or overnight with  $^{32}$ PO<sub>4</sub> as described previously [13]. Thereafter, erythrocytes were washed with PBS, resuspended to a hematocrit of 0.1% and exposed to different concentrations of lectins (0–100  $\mu$ g/ml) for 20 min at 20°C. At the low concentration of erythrocytes used, lectin-induced agglutination was not detected even at the highest lectin concentration tested. TPA (in DMSO) was added to a final concentration of

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*Abbreviations:* WGA, wheat germ agglutinin; Con A, concanavalin A; PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; DMSO, dimethylsulfoxide; DFP, diisopropyl fluoro-phosphate; PBS, 150 mM NaCl, 5 mM NaPO<sub>4</sub>, pH 7.6

1  $\mu\text{M}$  to the lectin-treated cell suspension (the final concentration of DMSO in the cell suspension was less than 0.1%). After a 10-min incubation at 37°C, the cells were centrifuged for 5 min at  $800 \times g$ ; controls were treated identically, with the omission of TPA. The cell pellet was solubilized directly in SDS-containing sample buffer and electrophoresed in a 10% polyacrylamide gel [18]. The gels were stained with Coomassie blue R-250 and the phosphoproteins were visualized by autoradiography. Quantitation of band 4.1, band 4.9 and spectrin phosphorylation was done by cutting out the appropriate bands from the gel and counting the gel pieces in a scintillation counter. In the case of band 4.1 and spectrin, the counts obtained were normalized to the amount of the respective proteins present in each lane determined by scanning densitometry of the Coomassie blue-stained gels.

### 2.3. Phosphorylation in ghosts

Red cells were washed in PBS and exposed to WGA and Con A as above. Half of the cells were treated with 1  $\mu\text{M}$  TPA at 37°C for 20 min in PBS, followed by three washes in PBS; controls were treated identically, with the omission of TPA. TPA treated and untreated cells were lysed at 4°C in lysis buffer (5 mM  $\text{NaPO}_4$ , pH 8.2, and 0.1 mM EGTA) containing 2 mM DFP. Ghosts were washed in the above buffer until white and were resuspended to a protein concentration of 0.5 mg/ml in 20 mM Tris-HCl, pH 7.6, containing 50  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (0.2  $\mu\text{Ci}/\mu\text{M}$ ) and 2 mM  $\text{MgCl}_2$ . Phosphorylation was carried out for 1 h at 37°C. At the end of the incubation, SDS-containing electrophoresis sample buffer was added to stop the reaction and to solubilize ghosts. Phosphorylation of the proteins was analyzed as above.

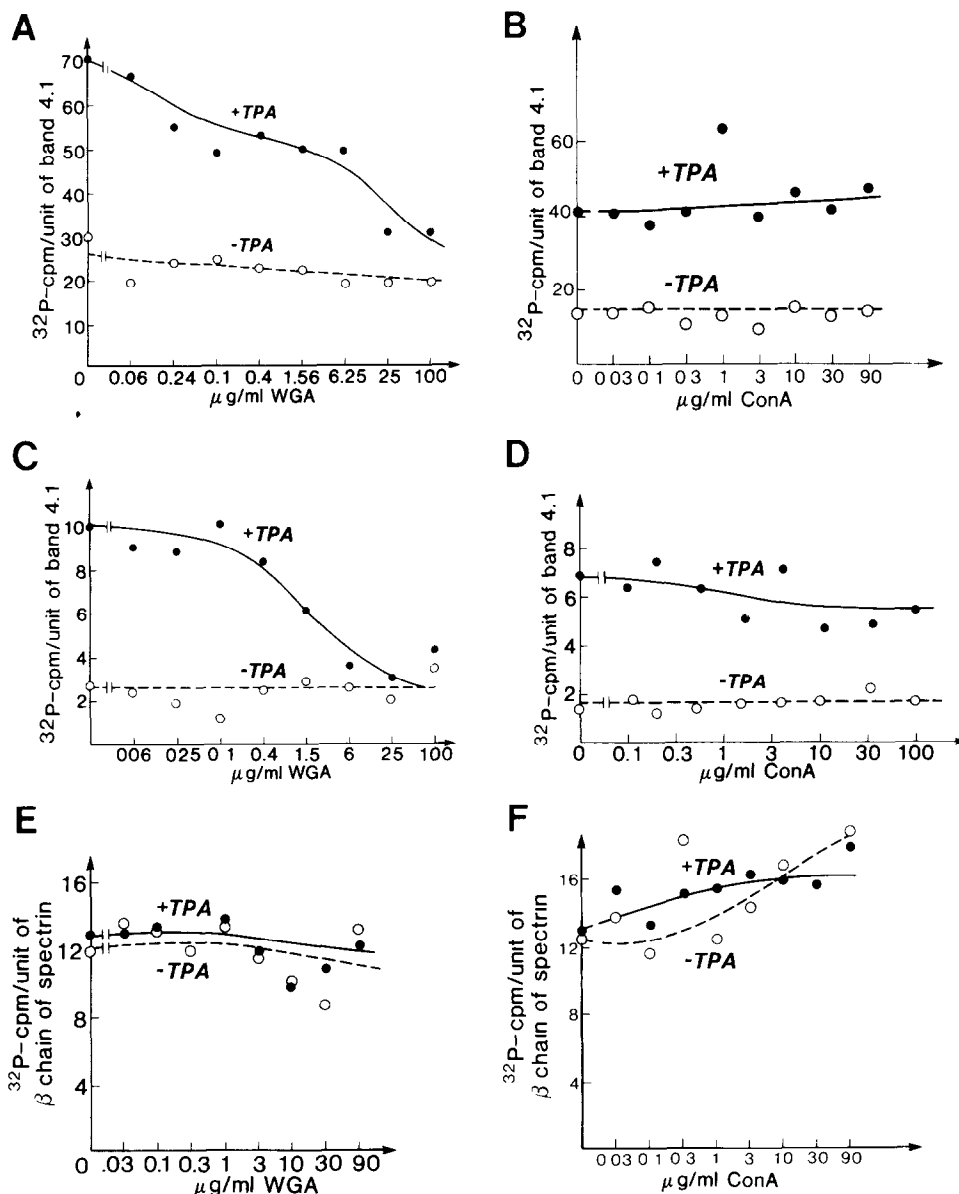


Fig. 1. Effect of TPA and lectins on phosphorylation of band 4.1 and spectrin in intact red blood cells or ghosts. (A) Intact red cells were incubated with  $^{32}\text{PO}_4$ , pretreated with the indicated concentrations of WGA, and treated or not treated with TPA as described in section 2. Band 4.1 phosphorylation was measured as described in section 2. (●) Phosphorylation in TPA-treated cells; (○) phosphorylation in non-TPA-treated cells. (B) Same experiment as in A, except cells were treated with increasing concentrations of Con A. (C) Ghosts were prepared from TPA-treated or control cells which had been pretreated with the indicated concentrations of WGA as described in section 2. Band 4.1 phosphorylation was quantified as described in section 2. (●) Ghosts from TPA-treated cells; (○) ghosts from control (non-TPA-treated) cells. (D) Same experiment as C except cells were treated with increasing concentrations of Con A. (E,F) Same experiments as C and D except spectrin phosphorylation was measured.

#### 2.4. Binding of $^{125}\text{I}$ -WGA and $^{125}\text{I}$ -Con A to red blood cells

WGA and Con A were labeled with  $^{125}\text{I}$ -labeled Bolton Hunter reagent according to the manufacturer's recommendations up to specific activity of 25–30  $\mu\text{Ci}/\text{mg}$  of protein. Red cells were washed as above, resuspended to  $5 \times 10^7$  cells/ml and exposed to the radiolabeled lectins at different concentrations (see legend, fig.2) for 20 min at 20°C. This was followed by an additional incubation for 10 min at 37°C with or without 1  $\mu\text{M}$  TPA. At the end of incubation, the cells were sedimented, washed with PBS and the bound radioactivity was measured in a gamma counter. The binding data are presented as number of moles of WGA and Con A per mole of glycophorin or band 3, respectively. The number of moles of lectins was computed assuming  $5 \times 10^5$  copies of glycophorin and  $10^6$  copies of band 3 per cell [17]. All experiments shown were performed at least three times.

### 3. RESULTS

Treatment of intact red blood cells with TPA causes the translocation of PKC from the cytosol to the plasma membrane, where it phosphorylates the membrane skeletal proteins band 4.1, band 4.9 and adducin [13,19,20]. Fig.1A,B show that treatment of intact red cells with TPA resulted in a 2–3-fold increase in  $^{32}\text{PO}_4$  incorporation into band 4.1 as a result of phosphorylation by protein kinase C. Fig.1A shows that addition of WGA to these cells resulted in a dose-dependent inhibition of TPA-stimulated band 4.1 phosphorylation. A similar WGA-dependent inhibition was detected for PKC-catalyzed phosphorylation of band 4.9 in intact cells (not shown). By contrast, fig.1B shows that Con A over the same concentration range had no effect on TPA-stimulated band 4.1 phosphorylation. As with band 4.1, Con A was without effect on TPA-stimulated band 4.9 phosphorylation (not shown). In several experiments similar to those shown in fig.1, the maximal WGA-dependent inhibition of phosphorylation ranged from 50 to 90% for band 4.1 and 60 to 70% for band 4.9. Neither WGA nor Con A changed the

background (PKC-independent) phosphorylation of either band 4.1 (fig.1A,B) or band 4.9 (not shown).

Similar experiments were done using ghosts prepared from cells which had been either treated or not treated with TPA and which had been pre-treated with increasing concentrations of lectins. Ghosts from TPA-treated red cells contain activated membrane-associated PKC [13,19] which phosphorylates bands 4.1, 4.9 and adducin. Fig.1C shows that when  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  was added to ghosts from TPA-treated cells, there was a 3–4-fold higher phosphorylation of band 4.1 than in ghosts from non-TPA-treated cells, but this difference was nearly abolished in ghosts from cells to which increasing concentrations of WGA above 0.1  $\mu\text{g}/\text{ml}$  were added. By contrast, pretreatment of the cells with Con A had only a small effect on PKC-stimulated phosphorylation (fig.1D). As in the above studies, similar effects of WGA were found with PKC phosphorylation of band 4.9 (not shown), and lectins had no effect on PKC-independent (i.e. in the absence of added TPA) phosphorylation of this protein.

Two lines of evidence suggest that the effects of WGA are specific for PKC-catalyzed phosphorylation. First, fig.1E shows that within the precision of our measurements, TPA was without effect on spectrin  $\beta$ -chain phosphorylation, suggesting that PKC is not involved in spectrin phosphorylation. Fig.1E,F shows that this PKC-independent phosphorylation of spectrin  $\beta$ -chain was unaffected by addition of either WGA or Con A to intact cells. Second, WGA was without effect on the basal, TPA-independent, phosphorylation of either band 4.1 or 4.9 (fig.1A–D).

In order to ensure that the difference in the effects of WGA and Con A on PKC phosphorylation were not due to differences in the amounts of each lectin bound to their respective receptors, we performed the experi-

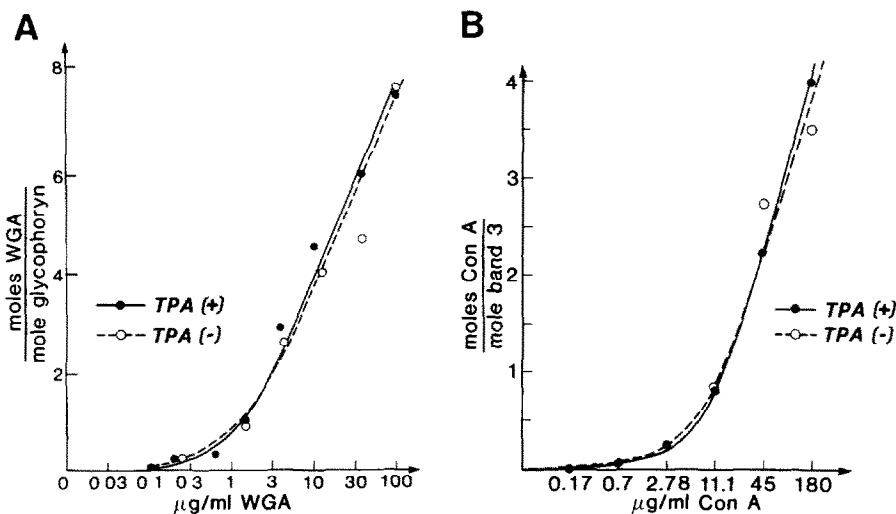


Fig.2. Binding of  $^{125}\text{I}$ -WGA and  $^{125}\text{I}$ -Con A to red blood cells. (A) Intact red cells which had been either treated (●) or not treated (○) with TPA were incubated with increasing concentrations of  $^{125}\text{I}$ -WGA, and binding was measured as described in section 2. (B) Same as A except  $^{125}\text{I}$ -Con A binding was measured.

ment shown in fig.2.  $^{125}\text{I}$ -WGA and  $^{125}\text{I}$ -Con A were used to quantify the number of lectin molecules bound per molecule of glyophorin or band 3, respectively. Fig.2A,B show that the number of lectin molecules bound per molecule of receptor was similar for both lectins over the concentration range studied, and that lectin binding to the cells was not affected by TPA treatment. Fig.2 also shows that the concentration of WGA at which half-maximal effects on band 4.1 phosphorylation were seen (about  $1\text{ }\mu\text{g/ml}$  WGA) correspond to approximately one WGA bound per glyophorin molecule.

#### 4. DISCUSSION

Previous studies have suggested that agents which bind to glyophorin A (or  $\alpha$ ) and possibly band 3 can perturb the membrane skeleton and affect red cell shape and mechanical properties [11,12,14]. The results presented here suggest that these effects are also reflected in changes in the state of phosphorylation of the key membrane skeletal protein band 4.1. Similar effects were seen on the skeletal proteins band 4.9 and adducin (data not shown), both prominent substrates for red cell PKC.

The mechanism responsible for the effects seen here remains to be determined. It may be that WGA affects the binding of or translocation of PKC to the membrane. Very little is known about the binding site for PKC on the red cell membrane other than that binding is stimulated by TPA and related compounds [21,22]. However, we have previously found that neuraminidase treatment of glyophorin, which affects skeletal phosphorylation by PKC in the same way as does WGA, has no effect on TPA-stimulated translocation of PKC to the membrane [13].

It is also possible that binding of WGA to glyophorin affects the conformation or accessibility of membrane skeletal proteins such that the phosphorylation by PKC is suppressed. This mechanism would require that band 4.1, 4.9 and adducin all respond similarly when WGA binds to glyophorin. Such an effect would imply a global reorganization of the skeletal network in response to WGA binding to glyophorin. This type of reorganization would not be inconsistent with the dramatic effects which WGA and antibodies to glyophorin have on membrane mechanical properties and skeletal organization cited above.

Finally, it is possible that changes in the activity of PKC are the cause of the skeletal reorganization induced by lectins or anti-glyophorin antibodies, rather than the result of such reorganization. While there are no known physiological circumstances under which red cell PKC is activated, the study of transmembrane regulation in the relatively simple membrane skeleton of the red cell may provide clues to understanding similar phenomena in more complex cells.

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